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Medical Sciences

An anti-angiogenic state in mice and humans with retinal photoreceptor cell degeneration

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Abstract

Abnormal angiogenesis accompanies many pathological conditions including cancer, inflammation, and eye diseases. Proliferative retinopathy because of retinal neovascularization is a leading cause of blindness in developed countries. Another major cause of irreversible vision loss is retinitis pigmentosa, a group of diseases characterized by progressive photoreceptor cell degeneration. Interestingly, anecdotal evidence has long suggested that proliferative diabetic retinopathy is rarely associated clinically with retinitis pigmentosa. Here we show that neonatal mice with classic inherited retinal degeneration (*Pdeb*^{rd1}/*Pdeb*^{rd1}) fail to mount reactive retinal neovascularization in a mouse model of oxygen-induced proliferative retinopathy. We also present a comparable human paradigm: spontaneous regression of retinal neovascularization associated with long-standing diabetes mellitus occurs when retinitis pigmentosa becomes clinically evident. Both mouse and human data indicate that reactive retinal neovascularization either fails to develop or regresses when

the number of photoreceptor cells is markedly reduced. Our findings support the hypothesis that a functional mechanism underlying this anti-angiogenic state is failure of the predicted up-regulation of vascular endothelial growth factor, although other growth factors may also be involved. Preventive and therapeutic strategies against both proliferative and degenerative retinopathies may emerge from this work.

Introduction

Excessive formation of new blood vessels in the retina is considered a hallmark of ischemic retinopathies such as diabetic retinopathy, a leading cause of blindness in the United States and Europe. Moreover, ocular neovascularization is considered a common etiological factor in diseases ranging widely in age of onset, from retinopathy of prematurity in oxygen-treated infants, to sickle cell disease and retinal venous occlusions seen in adults, to age-related macular degeneration observed in the elderly (1, 2).

The present investigation offers insight into the mechanism and possible prevention of retinal neovascularization. The study utilizes two well-established mouse models of disease. In the first, a mouse model of oxygen-induced ischemic retinopathy, mice are exposed to 75% oxygen (O_2) from postnatal day 7 (P7) to P12, after which time they are returned to room air. Their retinas are analyzed 5-9 days later (P17-P21), by which time neovascularization has supervened on the retinal surface (3). The exposure of neonatal mice to 75% O_2 causes vasoconstriction of the central retinal blood vessels. The decreased retinal perfusion, along with the return of the mice to room air, is believed to lead to a relative retinal tissue hypoxia and ischemia, resulting in marked retinal neovascularization (3-5).

The mechanism underlying neovascularization in this animal model and in certain human diseases is thought to involve, among other factors, a hypoxia-driven up-regulation of vascular endothelial growth factor (VEGF; refs. 4-10). Overexpression of VEGF in the retina is sufficient to cause intraretinal and subretinal neovascularization (8), whereas inhibition of VEGF expression or activity inhibits retinal neovascularization (9). VEGF, a 45-kDa glycoprotein that binds to several transmembrane tyrosine kinase receptors, is produced by glial cells of the neural retina, such as specialized astrocytes, including Muller cells, among other cell types (4-6). VEGF expression in the retina decreases within 6 h of exposure to 75% oxygen and remains decreased for the duration of the hyperoxia. In contrast, an increase in retinal VEGF expression is observed between 6 and 12 h after the return to room air, and such expression remains elevated during development of the neovascularization. Therefore, VEGF levels appear to play a dual role in this retinopathy model: a down-regulation of VEGF by hyperoxia induces blood vessel regression, whereas subsequent up-regulation of VEGF leads to retinal neovascularization (5, 10).

The second mouse model used in this study is the classic autosomal recessive inherited degenerative disease of photoreceptor cells known as retinal degeneration, $Pdeb^{rd1}$. This disease is caused by a nonsense mutation in the β subunit of the rod photoreceptor cell-specific phosphodiesterase (11-14).

Light absorption by rhodopsin activates transducin, a G protein, which in turn promotes cGMP hydrolysis by the specific phosphodiesterase, leading to hyperpolarization of rod photoreceptor cells (15). The widely distributed *Pdeb*^{rd1} mutation (11) can be traced back directly to Keeler's rodless mutation (16), as shown by analysis of DNA extracted from Keeler's original microscope slides 70 years later (17). The retinal development in *Pdeb*^{rd1}/*Pdeb*^{rd1} mice proceeds normally until P11. At that time, the development of photoreceptor cell outer segments arrests, and the rod cell nuclei, inner segments, and outer segments begin to degenerate. Photoreceptor cell degeneration then proceeds rapidly, and exceeds 80% by P15, and 90% by P21 (ref. 18). By P25-P30 only one sparsely populated row of photoreceptor cell nuclei remains, and the outer segments have disappeared. By the beginning of the fourth postnatal week, most surviving photoreceptor cells are cone cells (19, 20). Apoptosis of the photoreceptor cell is the final pathogenic event common to all animal models of retinal degeneration (21, 22). In addition to the primary photoreceptor cell loss, *Pdeb*^{rd1} mutant mice (23) and patients with retinitis pigmentosa (24) may also have an altered retinal blood flow.

Materials and Methods

Animals. This study adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Mouse experiments were approved by the Animal Care and Use Committees of the Burnham Institute and of the University of Texas M. D. Anderson Cancer Center. The strain of inbred congenic C57BL/6 mice carrying the *Pdeb*^{rd1} mutation has been described (25). We used mice at the F77N2F14-16 generations. C57BL/6 + /+ wt mice (Harlan, Indianapolis) were used as controls.

Induction of Retinal Neovascularization. P7 mouse pups with their nursing mothers were exposed to 75% oxygen for 5 days. Mice were returned to room air (20.8% O_2) on P12. For histological analysis, mice were killed between P17 and P21 and eyes were enucleated and fixed in 4% paraformaldehyde in PBS overnight at 4°C. For RNA isolation, mice were killed and their eyes were enucleated on P12 either immediately or 12 h after return to room air from 75% O_2. Retinas were dissected and stored in TRI reagent (Sigma) at −80°C.

Histological and Immunohistochemical Analysis. Fixed and alcohol dehydrated eyes were embedded in paraffin and serially sectioned at 5 µm. Tissue sections were stained either with hematoxylin and eosin (H&E) or immunostained with an anti-von Willebrand factor antibody (Dako) according to the manufacturer's instructions. Endothelial cell nuclei on the vitreous side of the internal limiting membrane were counted (3). At least six H&E-stained sections were evaluated per eye, and the average number of nuclei was counted from at least eight eyes for each experimental condition. Student's *t* test was used to determine whether the differences observed were statistically significant. All experiments were repeated at least three times under similar conditions.

Northern Blot Analysis and *in Situ* Hybridization. RNA was isolated from mouse retinas by using the TRI reagent according to the manufacturer's instructions. Total retinal tissue RNA (8 µg per sample)

from each time point was electrophoresed on a 1% agarose gel containing 6% formaldehyde (26). RNA was transferred to nylon membranes and hybridized with a ^{32}P-labeled VEGF$_{165}$ cDNA probe (26). Densitometry data were acquired and analyzed by using a FluorChem imager and software (Alpha Innotech, San Leandro, CA). Colorimetric *in situ* hybridization of paraffin-embedded eyes was performed with hyperbiotinylated oligoprobes (27).

Results

Abolishment of Reactive Retinal Neovascularization in Young Mice with Inherited Retinal Degeneration. To test the angiogenic response of the Pdeb^{rd1} mutant retinas in response to ischemia, we designed experiments with the mouse models of O_2-induced retinopathy and retinal degeneration simultaneously. Combination of the models produced the surprising finding that the reactive retinal neovascularization characteristic of normal young mice exposed to high O_2 levels, and observed in wild-type (wt) and heterozygous animals, failed to occur in *Pdeb^{rd1}* homozygotes. Neovascularization was quantified by counting vascular endothelial cell nuclei protruding into the vitreous space from at least six sections of 8-36 eyes in five independent experiments (Table 1). Extensive induction of retinal neovascularization (40.0 ± 3.2 endothelial cell nuclei per eye section) was seen in C57BL/6 +/+ wt mice on P17 after 75% oxygen treatment from P7 to P12 (Fig. 1*b*) and in heterozygous +/*Pdeb^{rd1}* mice (data not shown). Virtually no endothelial cell nuclei (0.4 ± 0.1 endothelial cell nuclei per eye section) were seen in the *Pdeb^{rd1}/Pdeb^{rd1}* retinas on P17 after exposure to 75% oxygen from P7 to P12 (Fig. 1*d*). At this time only a few layers of nuclei remained in the photoreceptor cell layer. Also, no endothelial cell nuclei were seen on or after P21, ruling out the possibility of delayed retinal neovascularization (data not shown). No endothelial cell nuclei were seen on P17 in either wt or *Pdeb^{rd1}/Pdeb^{rd1}* mice exposed only to room air (Fig. 1 *a* and *c*). Staining for von Willebrand factor confirmed that the cells protruding into the vitreous space of wt mice treated with 75% oxygen were indeed endothelial cells (Fig. 1 *e* and *g*) and that such cells were almost completely confined to the neural retina in *Pdeb^{rd1}/Pdeb^{rd1}* homozygotes (Fig. 1 *g* and *h*).

View this table: [in this window] [in a new window]	**Table 1.** Effect of O_2-induced retinal neovascularization in wt and *Pdeb^{rd1}*/Pdeb^{rd1} mice

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Fig. 1. Effect of relative hypoxia on C57BL/6 +/+ wt and on *Pdeb*^{rd1}/*Pdeb*^{rd1} mutant mouse retinas. (*a*) Wild-type retina on P17 of a mouse kept continuously in room air. (*b*) Wt retina on P17 after exposure to 75% oxygen for 5 days from P7 to P12. A large number of new blood vessels are seen protruding into the vitreous space. (*c*) *Pdeb*^{rd1}/*Pdeb*^{rd1} retina on P17 when mouse has been kept in room air. (*d*) *Pdeb*^{rd1}/*Pdeb*^{rd1} retina on P17 after exposure to 75% oxygen for 5 days from P7 to P12. No new blood vessels are seen protruding the vitreous space. (*e*) Anti-von Willebrand factor (vWF) antibody-immunostained section of wt retina on P17 after exposure to 75% oxygen from P7 to P12. (*f*) Detail of an H&E-stained section of a typical new blood vessel in a wt retina after exposure to 75% oxygen from P7 to P12. (*g*) vWF-antibody-stained section of *Pdeb*^{rd1}/*Pdeb*^{rd1} retina on P17 after exposure to 75% oxygen from P7 to P12. (*h*) Detail of an H&E-stained section of a *Pdeb*^{rd1}/*Pdeb*^{rd1} retina on P17 after exposure to 75% oxygen from P7 to P12. Arrows point to endothelial cell nuclei. (Scale bar: *a-d*, 100 µm; *e-h*, 35 µm.) ONL, outer nuclear layer.

Failure of the Predicted Up-Regulation of VEGF in Mice with Inherited Retinal Degeneration. VEGF has been suggested to be one of the key angiogenic factors in oxygen-induced retinal neovascularization (4-10). We hypothesized that differences in VEGF expression could play a role in the lack of neovascularization in the retinas of *Pdeb*^{rd1}/*Pdeb*^{rd1} mice and examined VEGF expression in retinal tissue by Northern blot analysis (Fig. 2). Total RNAs from wt and *Pdeb*^{rd1}/*Pdeb*^{rd1} mouse retinas were analyzed on P12 after exposing mice for 5 days to either 75% O_2 or room air. A decline in VEGF expression was seen during exposure to hyperoxia. This decrease was followed by a 150% increase in the VEGF expression in wt mouse retinas observed 12 h after the return to room air after 75% O_2 exposure, compared with that seen after exposure to room air only. In *Pdeb*^{rd1}/*Pdeb*^{rd1} mice, retinal VEGF expression remained low and unchanged even after exposure to 75% O_2 for 5 days, comparable to retinas of similar (otherwise isogenic) mice exposed only to room air. To determine whether inhibition of neovascularization was a consequence of an altered spatial expression pattern of VEGF rather than an overall alteration in VEGF expression levels in the *Pdeb*^{rd1}/*Pdeb*^{rd1} mouse retina, we analyzed VEGF expression in the retina by *in situ* hybridization. Tissue sections from wt and *Pdeb*^{rd1}/*Pdeb*^{rd1} mouse eyes were evaluated on P12, 12 h after of exposure to either 75% O_2 or room air for 5 days. Slightly higher VEGF mRNA levels were seen in the inner nuclear layer and in the inner plexiform layer of wt mouse retinas on P12, after 12 h in room air after 75% O_2 exposure. These expression patterns are consistent with previous studies (4), but a comparable increase in VEGF expression was not seen in any region in *Pdeb*^{rd1}/*Pdeb*^{rd1} mouse retinas after 75% O_2 exposure (data not shown).

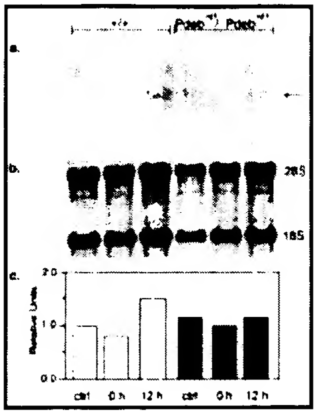

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Fig. 2. VEGF expression in wt mouse and *Pdeb^rd1^/Pdeb^rd1^* mouse retinas. (*a*) Northern blot analysis of VEGF expression in wt (lanes 1-3) and $Pdeb^{rd1}/Pdeb^{rd1}$ (lanes 4-6) mouse retina. Wild-type and $Pdeb^{rd1}/Pdeb^{rd1}$ mice were exposed to 75% O_2 from P7 to P12. On P12, retinal RNA was isolated immediately (0 h; lanes 2 and 5) or 12 h (lanes 3 and 6) after return to room air from 75% O_2. Retinal VEGF expression was quantified also from retinas of mice kept only in room air until P12 (control; lanes 1 and 4). Arrow indicates VEGF transcript (3800 bp). (*b*) 28S and 18S ribosomal markers serve as loading controls. (*c*) Integrated density values of VEGF transcripts shown in *a* and *b* were quantified. The baseline value for VEGF expression in wt mice kept only in room air until P12 was set to 1.0. Standard deviations were typically less than 10% of the mean. A representative experiment is shown.

Regression of Diabetic Retinopathy in Some Patients with Retinitis Pigmentosa. Are there clinical counterparts to this mouse experiment in which an exogenous stimulus of pathological formation of new retinal blood vessels fails in the presence of advanced photoreceptor cell degeneration? Here we present a clinical case in which proliferative retinopathy regressed spontaneously in a diabetic patient with concurrent retinitis pigmentosa. On fundus examination of a 36-year-old woman, diagnosed with type I diabetes mellitus for the past 34 years, we observed, in both eyes, inactive fibroglial membranes projecting into the vitreous space from the optic discs (Fig. 3). This pattern was consistent with regressed retinal neovascularization, often observed in cases of patients with proliferative diabetic retinopathy after successful laser treatment (15). However, this patient had never received laser treatment. In the periphery and midperiphery of the fundus, attenuated vasculature and atrophic retina with granular and bone spicule pigmentary changes were observed, consistent with a diagnosis of retinitis pigmentosa (Fig. 3) and confirmed by a virtually flat electroretinogram (i.e., less than 10 μV; data not shown). In nondiabetics with retinitis pigmentosa, spontaneous regression of optic disk neovascularization caused by an unknown mechanism can also occur (28). This example and other sporadic clinical case reports (28-30) suggest that, in the clinical setting of rod photoreceptor cell degeneration, proliferative retinopathies may fail to develop or regress early.

Fig. 3. Spontaneously regressed optic disk neovascularization (arrow) in a 36-year-old woman with concurrent type I diabetes mellitus and retinitis pigmentosa. Note the granular and "bone spicule-like" pigmentary changes in the retina (asterisks) consistent with a diagnosis of retinitis pigmentosa.

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Discussion

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The pathogenesis of neovascularization in ischemic retinopathies is best considered in the context of vasculogenesis in the normal developing retina. Blood vessels enter the back of the embryonic eye at the eye-cup stage and reach the vitreal surface via the choroidal fissure. This fissure closes around the developing optic nerve and the blood vessels close to the vitreal surface, which supply the innermost part of the central retina. As the retina expands during and after the fetal period, the vessels branch and grow radially outward toward the retinal periphery. Astrocytes lie in the avascular zone just ahead of the radially spreading vessels, and are thought to stimulate and control the direction of vessel growth by local release of VEGF (4-6, 15, 31). Astrocytes also grow inward into the inner plexiform and inner nuclear layers of the developing retina, and they stimulate the growth of immature vessels inward in their path. Once blood vessels mature to the stage, at which point they are invested with peri-endothelial cells ("pericytes"), they lose responsiveness to VEGF (32). Another set of blood vessels supplies the choroid, which is just external to the pigment epithelial layer. The pigment epithelial layer itself and the entire length of the photoreceptor cells, from their synaptic endings in the outer plexiform layer, through their nuclei in the outer nuclear layer, to their specialized inner and outer segments close to the pigment epithelium, are normally avascular. In experimental or clinical contexts in which retinal hypoxia induces VEGF expression, new blood vessels will form either on the inner neural retina in young subjects or, in some older subjects, from the choroid across the pigment epithelium (31). In contrast, when VEGF basal expression drops, endothelial cells undergo apoptosis (5) and retinal vasculature regresses, resulting in a reduced retinal blood supply.

Recently, Arden proposed (33) the intriguing hypothesis that the high oxygen consumption of dark-adapted rod cells is the driving force of inner retinal hypoxia, with subsequent VEGF production leading to retinal neovascularization in ischemic retinopathies. This hypothesis was indirectly supported by the observation that diabetic retinopathy rarely occurs in retinitis pigmentosa patients (28-30, 33, 34) and by the clinical success of panretinal photocoagulation, a treatment that destroys a large number of rod photoreceptor cells and reduces intraocular VEGF levels (35). Our findings show that degeneration of rod cells leads to a total lack of reactive retinal neovascularization, accompanied by a failure in the

expected VEGF up-regulation. Taken together, these observations of *Pdeb^rd1^/Pdeb^rd1^* mice and a human patient afflicted with both diabetes mellitus and retinitis pigmentosa provide direct experimental and mechanistic evidence in support of Arden's hypothesis (33) and suggest that VEGF is a primary link between rod cell numbers and retinal neovascularization. Indeed, it is tempting to speculate that reducing the metabolic rate of rod cells at critical time windows may improve the incidence of retinopathy of prematurity or perhaps slow the progression of diabetic retinopathy in adults. For instance, it is conceivable that increased exposure of premature neonates to light may reduce O_2 consumption by rod photoreceptor cells and retinal hypoxia, ultimately improving their retinopathy. Paradoxically, such reasoning challenges the current recommendation to decrease ambient light exposure in that setting, which has actually failed to prevent retinopathy of prematurity (36).

These observations notwithstanding, VEGF is not the only angiogenic mediator whose production is affected by changes in O_2 tension (37-42). Moreover, VEGF inhibitors and blockers can only partially halt angiogenesis in the retinopathy of prematurity model (9), and not all of patients with diabetic retinopathy show a rise in VEGF (35). Thus, the total absence of retinal neovascularization in homozygous *Pdeb*^{rd1} mice argues that the degeneration of photoreceptor cells may have further effects on angiogenesis that are not VEGF-mediated. Our data do not rule out a possible role for other angiogenic factors known to be regulated by hypoxia (37-40), such as transforming growth factor-β, insulin-like growth factor-1, placental growth factor, and interleukin-8, and more studies are needed to clarify these multiple interactions in the retina.

Finally, this work highlights the recent awareness that growth factors and inhibitors may be involved in coordinating neural and vascular components of the retina by functioning simultaneously as photoreceptor cell survival factors and endothelial cell regulators. Basic fibroblast growth factor (bFGF) is elevated in *Pdeb*^{rd1}/*Pdeb*^{rd1} mice several days before photoreceptor cell death (43), and intravitreous injection of bFGF delays the onset of photoreceptor cell degeneration in selected animal models (44). Pigment epithelium-derived factor (PEDF), which is encoded by a gene that is closely linked to the *Pdeb* locus (45), is a survival factor for photoreceptor cells (46) and has been proposed also to play an anti-angiogenic role in the retina (41). Given that PEDF concentration is highest in the matrix surrounding the photoreceptor cell layer (41, 47), which undergoes apoptosis in *Pdeb*^{rd1}/*Pdeb*^{rd1} mice (21, 22), one might expect that a loss of PEDF would be correlated with an increase rather than a decrease in retinal angiogenesis. In our system, preliminary data based on immunostaining failed to show a correlation between PEDF and neovascularization (data not shown) and suggest that PEDF does not play a major role in the phenomenon described here. However, it is possible that PEDF or other angiogenesis inhibitors are released during the photoreceptor cell apoptotic process, which may contribute to the lack of retinal neovascularization. Roles for additional factors are suggested by the dramatic but unexplained variations in timing of retinal capillary growth into the pigment epithelial cell layer among mutant mice, which share a similarity in timing of photoreceptor cell degeneration (48). Finally, the neurotransmitter dopamine has been recently shown to inhibit VEGF-induced angiogenesis (49). However, dopamine synthesis and utilization are known to be suppressed at least in some mouse models of retinal degeneration (50).

In summary, we show that ischemia-induced neovascularization of the retina is abolished in a mouse

strain with inherited photoreceptor cell degeneration. We also document that regression of established reactive retinal neovascularization caused by diabetes mellitus can occur in a subset of adult patients also afflicted with retinitis pigmentosa. This striking, previously unreported failure to mount a reactive retinal neovascularization response to potent exogenous stimuli is associated with an absence of the expected VEGF up-regulation in the retina. Our findings support the hypothesis that O_2 consumption by rod cells is a major driving force in ischemic retinal neovascularization and controls VEGF production. Additional trophic agents and cytokines are likely also to be involved in this complex biological phenomenon. Further characterization of this anti-angiogenic state in the retina may lead to therapeutic approaches against important eye diseases such as ischemic retinopathies and late complications of retinitis pigmentosa.

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Abbreviations

P*n*, postnatal day *n*; PEDF, pigment epithelium-derived factor; VEGF, vascular endothelial growth factor; wt, wild type; H&E, hematoxylin and eosin.

Footnotes

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Blockade of in vivo VEGF-mediated angiogenesis by antisense gene therapy: role of Flk-1 and Flt-1 receptors

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Angiogenesis, the formation of new blood vessels from preexisting ones, is a critical component of various pathologies such as tumor progression, rheumatoid arthritis, and retinopathies. Vascular endothelial growth factor (VEGF) is a mitogenic and chimiotactic factor capable of inducing angiogenesis through the activation of its receptors, fetal liver kinase-1 (Flk-1) and fms-like tyrosine kinase-1 (Flt-1), expressed on endothelial cells. The purpose of the present study was to assess if a treatment with antisense (AS) oligonucleotides directed against VEGF receptors Flk-1 or Flt-1 mRNA could prevent VEGF-mediated angiogenesis. With the use of miniosmotic pumps, phosphate-buffered saline, VEGF, or VEGF combined with AS-Flk-1, AS-Flt-1, or AS-scrambled oligonucleotides were released in mouse testis for 14 days. VEGF (1, 2.5, and 5 µg) increased the formation of new capillary blood vessels by 236, 246, and 287%, respectively. The combination of AS-Flk-1 or AS-Flt-1 (200 µg) to VEGF (2.5 µg) reduced by 87 and 85% the formation of new blood vessels, respectively, and the expression of their corresponding proteins. These data demonstrate the therapeutical potential of AS-Flk-1 or AS-Flt-1 to prevent VEGF-mediated angiogenesis in vivo.

antisense oligonucleotides; gene therapy

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NEOPLASIA

Angiogenesis in acute promyelocytic leukemia: induction by vascular endothelial growth factor and inhibition by all-*trans* retinoic acid

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Abstract

Recent studies indicate that angiogenesis is important in the pathogenesis of leukemias, apart from its well-established role in solid tumors. In this study, the possible role of angiogenesis in acute promyelocytic leukemia (APL) was explored. Bone marrow trephine biopsies from patients with APL showed significantly increased microvessel density and hot spot density compared with normal control bone marrow biopsies. To identify the mediators of angiogenesis in APL, quantitative and functional assays were performed using the NB4 APL cell line as a model system. Conditioned media (CM) from the NB4 cells strongly stimulated endothelial cell migration. CM from the NB4 cells contained high levels of vascular endothelial growth factor (VEGF) but not basic fibroblast growth factor (bFGF). Most important, the addition of neutralizing VEGF antibodies completely inhibited the ability of NB4 CM to stimulate endothelial cell migration, suggesting that APL angiogenesis is mediated by VEGF. The effect of all-*trans* retinoic acid (ATRA) on APL angiogenesis was then studied. ATRA therapy resulted in a decrease in bone marrow microvessel density and hot spot density. CM from ATRA-treated APL cells did not

stimulate endothelial cell migration. Finally, quantitative assays showed that ATRA treatment resulted in the abrogation of VEGF production by the NB4 cells. These results show that there is increased angiogenesis and VEGF production in APL and that ATRA therapy inhibits VEGF production and suppresses angiogenesis. The addition of specific antiangiogenic agents to differentiation therapy or chemotherapy should be explored. (Blood. 2001;97:3919-3924)

Introduction

Angiogenesis is an essential phenotype in growth and development,[1] wound healing,[2] and reproduction.[3-5] An inadequate amount of blood vessel growth contributes to ulcer formation,[6] whereas excessive angiogenesis contributes to a number of pathologic conditions including arthritis, psoriasis, and neoplasia.[7]

In a series of now classical experiments, Folkman and colleagues[8] demonstrated that solid tumors cannot grow any larger than 2 to 3 mm in diameter without being able to induce their own blood supply. Recent evidence suggests that angiogenesis is critical in the pathogenesis of numerous different hematologic malignancies, including acute lymphoblastic leukemia[9,10] acute myelogenous leukemia,[11-13] chronic lymphocytic leukemia,[14] chronic myeloid leukemia,[15] and multiple myeloma.[16-18]

The purpose of this study was to examine the role of angiogenesis in acute promyelocytic leukemia (APL). APL is distinct from other forms of acute myelogenous leukemia on clinical, morphologic, and molecular bases. The molecular hallmark of this disease is the presence of a balanced reciprocal translocation involving the retinoic acid receptor α (*RAR α*) gene on chromosome 17 in all patients.[19-23] This type of leukemia has been extensively studied and is amenable to molecular analysis because of the availability of excellent model systems that recapitulate the disease.[24-28]

We also wanted to determine the possible effect of all-*trans* retinoic acid (ATRA) on angiogenesis in APL. ATRA therapy has brought about significant improvement in the remission rates of patients with APL[29,30] and is effective, at least in part, by inducing differentiation of the abnormal promyelocytes. We had previously shown that ATRA is antiangiogenic in oral squamous cell carcinoma,[31,32] and we wanted to assess whether ATRA therapy also has an antiangiogenic effect in APL.

Our results demonstrate that angiogenesis is increased in APL and is mediated by vascular endothelial growth factor (VEGF). In addition, we report that ATRA therapy inhibits VEGF production and is accompanied by a decrease in microvessel density.

Patients, materials, and methods

Patients

Each patient included in this study (n = 12; 8 women, 4 men) had a confirmed diagnosis of APL by either conventional cytogenetics showing the t(15;17) translocation or by polymerase chain reaction for the PML-RARα fusion protein. The mean age was 47.3 years. Of the 12 original patients, bone marrow biopsy specimens were available for 7 patients after ATRA treatment. All 7 patients were in morphologic remission. Of these 7 patients, 4 also underwent concurrent induction chemotherapy. Post-ATRA bone marrow biopsies were taken 23 to 93 days after diagnostic biopsies. Control bone marrow biopsy specimens (n = 12) were obtained from patients undergoing staging biopsy for breast carcinoma and lymphoma and from patients evaluated for anemia and thrombocytopenia. The mean age of the control patients was 52.8 years (8 women, 4 men).

Measurement of microvessel density

The degree of angiogenesis in the APL specimens was quantified by measuring microvessel density in bone marrow trephine sections of patients with newly diagnosed disease (n = 12). Vessel presence in tissue sections was highlighted by the use of standard immunohistochemistry techniques[33] using antibodies to CD34 (Immunotech, Westbrook, ME). Any endothelial cell cluster distinct from other endothelial cells, nonendothelial cells, and connective tissue was counted as a microvessel. It was unnecessary for a lumen to be present to define a microvessel. The entire bone marrow core section was examined, and microvessel density was quantified as the average number of microvessels per high-power field (600 ×). Hot spot density was measured by examining the bone marrow core section at low power (100 ×) and identifying the area with the highest microvessel density. Microvessel density in this area was then measured at high magnification (600 ×). In addition, expression of the angiogenic peptide VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) in bone marrow biopsies was assessed using standard immunohistochemistry techniques.[33]

Cell culture, ATRA treatment, and collection of conditioned media

The NB4 APL cells (obtained as a kind gift from Dr Peter Domer, University of Chicago) were cultured in RPMI 1640 with 10% heat-inactivated fetal calf serum and 1% penicillin-streptomycin and maintained at 37°C and 5% CO_2. ATRA (Sigma Pharmaceuticals, St Louis, MO) was prepared in dimethyl sulfoxide and stored as stock solution of 10^{-1} M at −80°C. ATRA was then added directly to the culture medium of the NB4 cells, with a final concentration of 10^{-6} M. NB4 cells were cultured in ATRA for 3 days before conditioned media (CM) were generated. Serum-free CM was generated

from ATRA-treated and untreated cells by rinsing the cells in RPMI 3 times, incubating the cells in RPMI for 4 hours, refeeding the cells with RPMI, and collecting the CM after 24 hours. Levels of the angiogenic peptides basic fibroblast growth factor (bFGF) and VEGF in the CM were quantified using the Quantikine bFGF and VEGF assays (R&D Systems, Minneapolis, MN), according to the manufacturer's protocols. For endothelial cell migration assays, CM were concentrated using Centriprep-3 concentrators (Amicon, Beverly, MA), and protein concentration was determined using the Coomassie protein assay reagent 23200 (Pierce Biochemical, St Louis, MO).

Endothelial cell migration assays

Endothelial cell migration assay was performed as previously described.[31] Briefly, human dermal microvascular endothelial cells (Cell Systems, Kirkland, WA) were starved overnight in endothelial basal media (Clonetics, San Diego, CA) containing 0.1% bovine serum albumin (BSA), harvested, resuspended into endothelial basal media with 0.1% BSA, plated on the bottom side of a modified Boyden chamber (Neuro Probe, Gaithersburg, MD), and allowed to attach in an inverted chamber at 37°C for 2 hours. The chamber was then re-inverted, and the test substances were added to the wells of the upper chamber (1 µg protein/well in a volume of 50 µL). After 4 hours the membranes were stained, and the number of endothelial cells that migrated to the upper chamber were counted per 10 high-power fields. Background migration was detected by using BSA alone, and purified growth factors (VEGF, 100 pg/mL; bFGF, 15 ng/mL) were used a positive controls. Neutralizing antibodies were used at concentrations previously optimized by dose-response experiments (anti-VEGF, 20 µg/mL; anti-bFGF, 20 µg/mL). All growth factors and antibodies were purchased from R&D Systems. The addition of neutralizing antibodies alone did not affect endothelial cell migration. Neutralizing antibodies completely inhibited the ability of the purified growth factors to stimulate migration.

Statistical analyses

Statistical analyses were performed using the Student *t* test. Differences were considered statistically significant when $P < .05$. Statistical analyses were performed using the Prophet 5.0 program (BBN Systems and Technologies, Cambridge, MA).

Results

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Increased angiogenesis in APL bone marrow biopsy specimens

Diagnostic bone marrow biopsy specimens from patients with APL had significantly higher microvessel density than normal control marrows (Figure 1). Mean microvessel

density in APL marrows was 7.0/high-power field (hpf), whereas in normal control marrow it was 2.4/hpf (P = .0001, Figure 2A). In addition, we examined hot spot density in the APL and control biopsies. Hot spot is defined as the high-power field with the highest microvessel density. An area of high microvessel density, such as a hot spot, may represent the emergence of an angiogenic clone and may be responsible for spread of the disease. Hot spot density was also significantly higher (P = .0007; Figure 2B) in APL marrows (15.5/hot spot) than in normal marrows (7.4/hot spot)

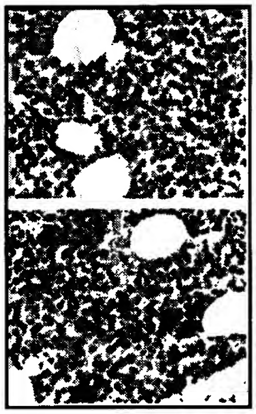

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Figure 1. Microvessels in APL and control bone marrow biopsy specimens. (A) Many microvessels were evident in this diagnostic bone marrow trephine biopsy specimen from a patient with APL. (B) In contrast, this normal control bone marrow biopsy sample had few microvessels. Microvessels were highlighted by immunohistochemistry with the use of antibodies to CD34 (brown). Original magnification, 600 ×.

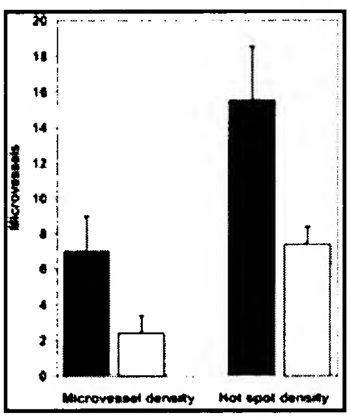

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Figure 2. Microvessel density and hot spot density in APL and control bone marrow biopsy specimens. Microvessel density in diagnostic APL bone marrow biopsy specimens (■, n = 12) was 7.0/hpf, whereas microvessel density in normal control bone marrow biopsy samples (□, n = 12) was 2.4/hpf (P = .0001). Hot spot density in APL bone marrow biopsy specimens (15.5/hot spot) was significantly (P = .0007) higher than in control bone marrow biopsy specimens (7.4/hot spot)

Angiogenesis in APL is mediated by VEGF

Whether angiogenesis occurs in a particular tissue depends on the balance between the relative amounts of molecules that induce and molecules that inhibit angiogenesis.[34] We

used the NB4 cell line as a model system to study the possible inducers or suppressors of APL angiogenesis. The NB4 cell line was established from a patient with APL and has all the characteristic features of APL including the t(15;17) translocation.[35] To assess the angiogenic potential of the NB4 cells, the conditioned media (CM) from these cells were tested in the endothelial cell migration assay. CM from NB4 cells strongly stimulated endothelial cell migration (Figure 3A). To identify the possible mediators of angiogenesis within the NB4 CM, we performed quantitative assays for the angiogenic peptides basic fibroblast growth factor (bFGF) and VEGF. The CM from the NB4 cells contained high levels of VEGF but not bFGF (Table 1). These data suggested that NB4 cells induced endothelial cell migration through the production of VEGF. However, there are at least 18 known inducers of angiogenesis,[36] and it is possible that substances other than VEGF are involved in stimulating migration. To test this possibility, we used neutralizing antibodies to VEGF and bFGF in the endothelial cell migration assays. The addition of anti-VEGF neutralizing antibodies completely inhibited endothelial cell migration, indicating that VEGF is a major mediator of angiogenesis (Figure 3A). As was expected, the addition of anti-bFGF antibodies had no effect on endothelial cell migration (Figure 3A).

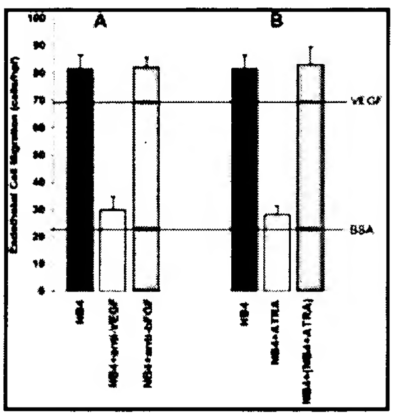

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Figure 3. VEGF-mediated stimulation of endothelial cell migration by NB4 CM. (A) Conditioned media from NB4 cells strongly stimulated endothelial cell migration. Migration was completely inhibited by the addition of antibodies to VEGF. The addition of anti-bFGF antibodies had no effect on endothelial cell migration. The upper horizontal line is a positive control using purified VEGF. The lower horizontal line is a negative control using bovine serum albumin. (B) In contrast to CM from untreated NB4 cells, CM from NB4 cells treated with ATRA did not stimulate endothelial cell migration. The addition of CM from NB4 cells treated with ATRA to CM from untreated NB4 cells did not result in the inhibition of endothelial cell migration.

Table 1. Production of angiogenic peptides by NB4 cells

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ATRA therapy inhibits VEGF production and angiogenesis in vitro and in vivo

We then examined angiogenesis in bone marrow biopsy samples from patients in

morphologic remission after ATRA therapy. ATRA-treated bone marrows had fewer microvessels than the diagnostic bone marrow samples (Figure 4). Quantification (Figure 5A) revealed a significant decrease (P = .0063) in microvessel density in bone marrow samples from APL patients treated with ATRA (4.2/hpf) in comparison to their corresponding diagnostic bone marrow biopsy samples (8.1/hpf). Hot spot density (10.4/hot spot) was also lower in the ATRA-treated bone marrow samples than in the corresponding diagnostic bone marrow samples (17.3/hot spot; P = .0159).

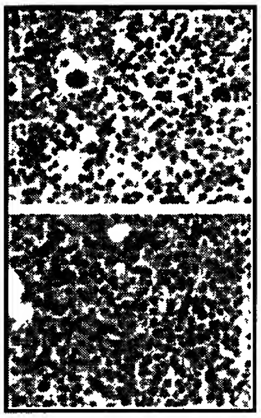

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Figure 4. Microvessels in APL bone marrows at diagnosis and remission. Bone marrow trephine section from an APL patient in ATRA-induced remission (A) had fewer microvessels than a diagnostic bone marrow biopsy specimen from the same patient (B). Microvessels were highlighted immunohistochemically with the use of antibodies to CD34 (brown). Original magnification, 600 ×.

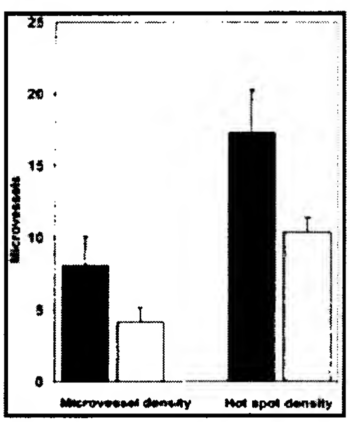

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Figure 5. Microvessel density and hot spot density in APL bone marrows at diagnosis and remission. Microvessel density in bone marrow biopsy specimens obtained from patients (n = 7) in ATRA-induced remission (□, 4.2/hpf) was significantly decreased (P = .0063) compared with microvessel density (8.1/hpf) in diagnostic bone marrow biopsy samples from the same patients (■). Hot spot density in bone marrow biopsy specimens from APL patients in ATRA-induced remission (10.4/hot spot) was also significantly (P = .0159) lower than hot spot density (17.3/hot spot) in their corresponding diagnostic bone marrow biopsy specimens.

To study the possible role of ATRA on APL angiogenesis in our in vitro system, we then treated the NB4 cells with ATRA and assayed its ability to stimulate endothelial cell migration. CM from ATRA-treated APL cells did not stimulate endothelial cell migration (Figure 3B). In addition, mixing CM from NB4 cells and ATRA-treated NB4 cells did not

inhibit migration (Figure 3B), indicating that the loss of angiogenic potential by NB4 cells resulted from the loss of production of an inducer of angiogenesis rather than from the production of an inhibitor of angiogenesis. Quantitative assays showed that ATRA treatment resulted in the abrogation of VEGF production by the NB4 cells (Table 1).

Finally, to confirm the relevance of our results in vivo, we performed VEGF immunostaining in the APL bone marrow biopsy samples. Figure 6 shows that the tumor cells of the diagnostic bone marrow biopsy specimens were positive for VEGF and that ATRA treatment resulted in a marked decrease in VEGF staining.

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Figure 6. VEGF staining in APL bone marrows at diagnosis and remission. (A) Diagnostic bone marrow biopsy sample from a patient with APL showed strong VEGF staining in the tumor cells. (B) Bone marrow biopsy sample from the same patient obtained after remission induced by ATRA therapy showed decreased VEGF staining intensity.

Discussion

We have demonstrated increased angiogenesis in the bone marrow of patients with APL. Both microvessel density and hot spot density were higher in diagnostic bone marrow biopsy specimens from patients with APL than in normal control bone marrow. These findings are consistent with increased bone marrow angiogenesis observed in numerous types of acute and chronic leukemia and in multiple myeloma. In APL, abnormal promyelocytes proliferate in the bone marrow, and increased angiogenesis may support this proliferation. Bone marrow angiogenesis may also facilitate the spread of leukemic cells beyond the bone marrow. The experiments presented here suggest that VEGF is a major mediator of angiogenesis in APL. Cultured NB4 APL cells produced VEGF, and APL cells in bone marrow biopsy samples were positive for VEGF. Conditioned media from NB4 cells stimulated endothelial cell migration; this migration was completely inhibited by anti-VEGF antibodies.

In addition to stimulating the production of new blood vessels, VEGF may be involved in complex autocrine and paracrine interactions in the bone marrow microenvironment. One possibility is that there may be paracrine interactions in which VEGF produced by APL cells promotes endothelial cell migration and proliferation and that the endothelial cells produce factors that promote tumor cell growth. This paracrine interaction would result in a positive feedback loop that may enhance both angiogenesis and tumor cell proliferation. Other examples of this type of paracrine loop occur in leukemias. Paracrine pathways involving leukemic cells have been described for tumor necrosis factor-α[37] and interleukin (IL)-1.[38] In addition, Fiedler et al[12] found that VEGF stimulated the production of granulocyte macrophage colony stimulating factor (GM-CSF), and Bellamy et al[39] reported that VEGF can stimulate M-CSF, G-CSF, IL-6, and stem cell factor production in human umbilical vein endothelial cells. Each of these cytokines could potentially elicit growth-stimulatory signals on leukemic cells. Alternatively, it is possible that VEGF may act in an autocrine fashion[12,40] and have some positive biologic effects on the leukemic cells themselves. In APL, there may also be a link between angiogenesis and the severe coagulopathies that accompany the disease. An important mediator of the coagulation abnormalities in APL is tissue factor.[41] VEGF has been shown to increase tissue factor expression[42,43] and may, therefore, be responsible, at least in part, for the disordered coagulation by acting in an autocrine fashion on the APL cells.

ATRA therapy results in a decrease in microvessel density, hot spot density, and VEGF reactivity in the bone marrow of patients with APL. NB4 cells treated with ATRA do not stimulate endothelial cell migration because of suppression of VEGF production. Previously, we had demonstrated that retinoic acid can modulate expression of the angiogenic phenotype through 2 different pathways. ATRA induces squamous cell carcinoma tumor cells to produce a retinoic acid-inducible inhibitor of angiogenesis.[32] In addition, it can cause endothelial cells to become refractory to inducers of angiogenesis.[31] The inhibition of VEGF secretion by ATRA represents a third possible mechanism by which retinoids modulate tumor-induced angiogenesis.

ATRA induces the differentiation of abnormal promyelocytes into neutrophils. This differentiation takes place through multiple mechanisms, including degradation of the PML-RARα protein, transcriptional activation of the wild-type *RAR*α gene, and nuclear relocalization of PML and other proteins.[44] The reduction of VEGF production on ATRA treatment could be part of the differentiation process that results in the conversion of the abnormal promyelocytes into mature neutrophils. An alternative explanation is that ATRA directly down-regulates APL tumor cell expression of VEGF, independent of the differentiation pathway. Direct down-regulation of VEGF production after retinoic acid treatment has been observed in human keratinocytes[45] and is dependent on the anti-AP-1 activity of retinoic acid.[46]

Recent improvements in the treatment of APL have led to 4-year disease-free survival rates greater than 70%.[47-49] However, toxic effects of induction therapy (including retinoic

acid syndrome) and relapse remain the major obstacles to cure.[48,50] A possible strategy to improve outcomes would be to use retinoids for their differentiating and antiangiogenic activities in conjunction with specific antiangiogenic agents such as anti-VEGF antibodies. The use of multiple inhibitors of angiogenesis to decrease toxicity while maintaining efficacy has been demonstrated in a number of different settings.[51-54] Because VEGF stimulates tissue factor production, anti-VEGF therapy may also aid in ameliorating the coagulopathies seen in APL. Although our results suggest that the angiogenesis paradigm may be extended to APL, they do not show that angiogenesis is essential in APL. Prospective clinical trials using specific antiangiogenic drugs, alone or in combination with existing therapeutic modalities, are necessary to assess the importance of angiogenesis in APL and other types of leukemia.

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Footnotes

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